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(54) Title: OLIGONUCLEOTIDE ANALOGS WITH NOVEL LINKAGES

(57) Abstract

The instant invention is directed to oligonucleotide compounds wherein on or more phosphodiester linkages between adjacent nucleotides are replaced by a formacetal/ketal type linkage. The nucleotides of the present invention are resistant to nucleases and do not need to exhibit the diastereomerism characteristic of many other oligonucleotide compounds and thus are capable of strong hybridization to target RNA or DNA. These oligonucleotide compounds are useful in therapies which modulate gene expression using antisense or other specifically binding oligomers.

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OLIGONUCLEOTIDE ANALOGS WITH NOVEL LINKAGES

Technical Field

The invention relates to the construction of modified oligonucleotides and their components which are resistant to nucleases, have enhanced ability to penetrate cells, and are capable of binding target oligonucleotide sequences *in vitro* and *in vivo*. The modified oligonucleotides of the invention are particularly useful in therapies utilizing antisense DNAs to interrupt protein synthesis or otherwise inactivate oligonucleotides. More specifically, the invention concerns oligomers which contain at least one replacement of the phosphodiester linkage with a formacetal/ketal type linkage bearing sufficiently stabilizing electron withdrawing groups.

Background Art

A rational basis for the development of antisense therapies for diseases whose etiology is characterized by, or associated with, specific DNA or RNA sequences has been recognized for a number of years. The basic principle is straightforward enough. The ultimate therapeutic agent, whether directly administered or generated *in situ*, is an oligomer which will be complementary to a DNA or RNA needed for the progress of the disease. By specifically binding to this target DNA or RNA, the ordinary function of these materials is interdicted.

The logic behind this approach is readily seen in visualizing the administration of, for example, an

oligomer having a base sequence complementary to that of an mRNA which encodes a protein necessary for the progress of the disease. By hybridizing specifically to this RNA, the synthesis of the protein will be interrupted. However, it is also possible to bind even double-stranded DNA using an appropriate oligomer capable of effecting the formation of a specific triple helix by inserting the administered oligomer into the major groove of the double-helical DNA. The elucidation of the sequences which form the targets for the therapeutics is, of course, a problem which is specific to each target condition or disease. While the general principles are well understood and established, there remains a good deal of preliminary sequence information required for the design of a particular oligomeric probe.

One feature of the oligomeric probes which is, however, common to most targets is the structuring of the backbone of the administered oligomer so that it is resistant to endogenous nucleases and is stable in vivo, but also retains its ability to hybridize to the target DNA or RNA. (Agarwal, K.L., et al., Nucleic Acids Res (1979) 6:3009; Agarwal, S., et al., Proc Natl Acad Sci USA (1988) 85:7079.) As nucleases attack the phosphodiester linkage, a number of modified oligonucleotides have been constructed which contain alternate linkages. Among these have been methylphosphonates (wherein one of the phosphorous-linked oxygens has been replaced by methyl) phosphorothioates (wherein sulphur replaces one of these oxygens) and various amidates (wherein NH_2 or an organic amine derivative, such as morpholidates or piperazidates, replace an oxygen). These substitutions confer enhanced stability, for the most part, but suffer from the drawback that they result in a chiral phosphorous in the linkage, thus leading to the formation of 2^n diastereomers where n is the number of modified

diester linkages in the oligomer. The presence of these multiple diastereomers considerably weakens the capability to hybridize to target sequences. Some of these substitutions also retain the ability to support a negative charge; the presence of charged groups decreases the ability of the compounds to penetrate cell membranes. (Miller, P.S., et al., Biochemistry (1981) 20:1874; Marcus-Secura, C.J., et al., Nucleic Acids Res (1987) 15:5749; Jayaraman, K., et al., Proc Natl Acad Sci USA (1981) 78:1537.) There are numerous other disadvantages associated with these modified linkages, depending on the precise nature of the linkage.

It has also been suggested to use carbonate diesters; however, these are highly unstable, and the carbonate diester link does not maintain a tetrahedral configuration exhibited by the phosphorous in the phosphodiester. Similarly, carbamate linkages, while achiral, confer trigonal symmetry and it has been shown that poly dT having this linkage does not hybridize strongly with poly dA (Coull, J.M., et al., Tet Lett (1987) 28:745; Stirchak, E.P., et al., J Org Chem (1987) 52:4202.

Of relevance to the present invention are modified phosphodiester linkages involving sulfur, rather than oxygen as a linking substituent. See, for example, Cosstick, R., et al., Tet Lett (1989) 30:4693-4696, which describes oligonucleotides containing 3'-thiothymidine.

The general approach to constructing oligomers useful in antisense therapy has been reviewed, for example, by van der Krol, A.R., et al., Biotechniques (1988) 6:958-976, and by Stein, C.A., et al., Cancer Res (1988) 48:2659-2668, both incorporated herein by reference.

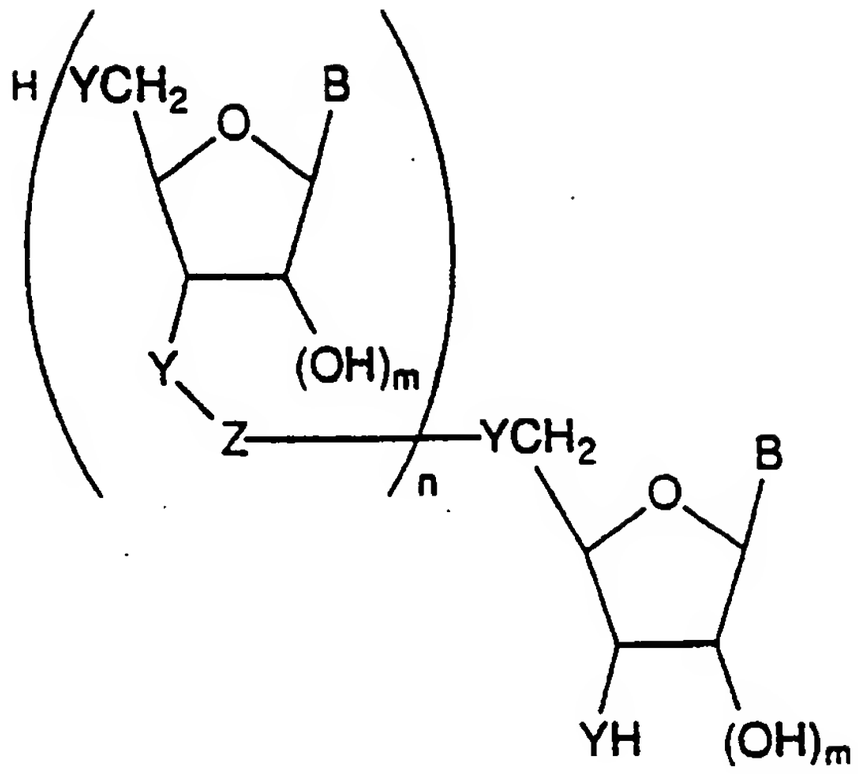
The present invention provides an oligomeric linkage which is isosteric to the phosphodiester found in

the native molecule, but which is resistant to nuclease digestion, and which is stable under physiological conditions, and which can be neutral so as to enhance cell permeation. Furthermore, the linkages can be achiral and thus do not lead to the problem of multiple diastereomers in the resulting compounds.

Disclosure of the Invention

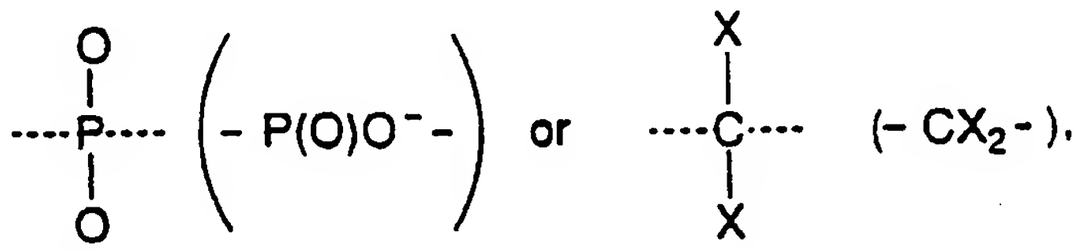
The invention is directed to modified oligonucleotides, including dimers and trimers, wherein the modification comprises replacing at least one phosphodiester linkage of the oligonucleotide with a formacetal/ketal type linkage of the formula YCX_2Y wherein each Y is, independently, O or S. Although each X can be chosen independently, it is preferred that both X are identical so that the carbon is achiral. In order to provide adequate stability, X should be a substituent which is stabilizing to the linkage, as opposed to, for example, the conventional electron donating substituents. The requirements for X differ depending on the nature of each Y, as is further explained below.

In one aspect, the invention is directed to oligonucleotides and intermediates in their synthesis which contain, in place of at least one phosphodiester or other conventional linkage, a linkage of the formula $-YCX_2Y-$ wherein each Y is independently O or S and wherein each X attached to the same carbon is independently chosen, but preferably both X are identical. X provides sufficient electron withdrawal to stabilize the linkage, as required by the formacetal/ketal linkage $-OCX_2O-$, the mixed linkages $-SCX_2O-$ and $-OCX_2S-$ or the dithio analog $-SCX_2S-$. Stated another way, the invention is directed to compounds of the formula



Formula 1

and their derivatized forms, as defined below, wherein each B is independently a purine or pyrimidine residue or their analogous forms, as defined below, wherein each Y is independently O or S, and wherein each Z is independently



or is a conventional phosphodiester substitute (including but not limited to $-P(O)S-$, $-P(O)NR_2-$, $-P(O)R-$, or $-P(O)OR'-$; $-CO-$ or $-CNR_2-$ wherein R is H or alkyl (1-6C) and R' is alkyl (1-6C)),

wherein X is as above defined, and

each m is independently 0 or 1, n is 1-200.

At least one Z must be CX_2. In additional aspects, the invention is directed to intermediates in

the synthesis of the compounds of the invention and to compositions for and methods to use these compounds in therapies which rely on specific binding to target oligonucleotides ("antisense" therapies).

Brief Description of the Drawings

Figure 1 shows an outline of, and idealized results of, the footprint assay for DNA-duplex binding.

Modes of Carrying Out the Invention

A. Definitions

As used herein, "antisense" therapy refers to administration or in situ generation of DNA or RNA oligomers or their derivatives which bind specifically to a target nucleic acid sequence. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" refers to the range of techniques generally employed under this description in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

"Oligomers" or "oligonucleotides" includes sequences of more than one nucleotide and specifically includes short sequences such as dimers and trimers.

"Derivatives" of the oligomers include those conventionally recognized in the art. For instance, the oligonucleotides may be covalently linked to various moieties such as intercalators, substances which interact specifically with the minor groove of the DNA double helix and other arbitrarily chosen conjugates, such as labels (radioactive, fluorescent, enzyme, etc.). These additional moieties may be (but need not be) derivatized through the formacetal/ketal type linkage as part of the

linkage itself. For example, intercalators, such as acridine can be linked through an $-Y-CH_2-Y-$ attached through any available -OH or -SH, e.g., at the terminal 5' position of RNA or DNA, the 2' positions of RNA, or an OH or SH engineered into the 5 position of pyrimidines, e.g., instead of the 5 methyl of cytosine, a derivatized form which contains $-CH_2CH_2CH_2OH$ or $-CH_2CH_2CH_2SH$ in the 5 position. A wide variety of substituents can be attached, including those bound through conventional linkages. Accordingly the indicated -OH moieties in the oligomer of formula (1) may be replaced by phosphonate groups, protected by standard protecting groups, or activated to prepare additional linkages to other nucleotides, or may be bound to the conjugated substituent. The 5' terminal OH is conventionally phosphorylated; the 2'-OH or OH substituents at the 3' terminus may also be phosphorylated. The hydroxyls may also be derivatized to standard protecting groups. In addition, specifically included are the 2'-derivatized forms of the nucleotide residues disclosed in copending U.S. serial no. (Atty. docket 4610-0003) filed on even date herewith, assigned to the same assignee, and incorporated herein by reference. The phosphodiester linkage shown may be replaced by alternative linking groups as long as one linkage is of the form $-YCX_2Y-$. These alternative linking groups include, but are not limited to embodiments wherein "Z" is P(O)S, $P(O)NR_2$, P(O)R, P(O)OR', CO, or CNR_2, wherein R is H or alkyl (1-6C) and R' is alkyl (1-6C). Not all Z in the same oligomer need to be identical.

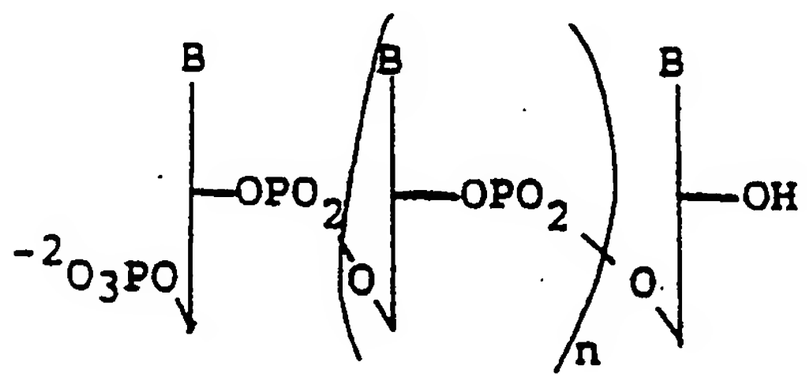
"Analogous" forms of purines and pyrimidines are those generally known in the art, many of which are used as chemotherapeutic agents. An exemplary but not exhaustive list includes 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil,

5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine. A particularly preferred analog is 5-methylcytosine.

The invention is directed to new compounds which are useful in antisense-based therapy and intermediates in their production, as well to methods to synthesize these compounds and their intermediates. In general, the invention compounds show enhanced stability with respect to nucleases by virtue of their modified linkages, as well as enhanced ability to permeate cells. Desirably, multiple phosphodiester linkages in the unmodified oligonucleotides are substituted by corresponding formacetal/ketal linkages or their thio-containing analogs. Though it is preferred that these substituent linkages be non-chiral in nature to enhance the ability to hybridize, useful compounds of the invention include those where each X is chosen independently. Thus chiral forms may also be used, and where multiple formacetal/ketal or analogous thio-linked linkages occur in the oligonucleotide, a number of embodiments of X- may be included.

As stated above, the linking moiety $-YCX_2Y-$, may be a formacetal/ketal or one or both of the oxygens may be replaced by a sulfur. The replacement of one or both oxygens by a thiol linkage may be advantageous in that the length of the carbon-sulfur bond corresponds more closely to the length of the phosphorus-oxygen bond in the native oligonucleotide. Furthermore, greater scope is possible in connection with the substituent X groups as further described below, since the thio analogs are more stable to hydrolysis, especially under acidic conditions. In addition, the preparation reactions may proceed more smoothly due to the enhanced nucleophilicity of the SH group in the intermediates.

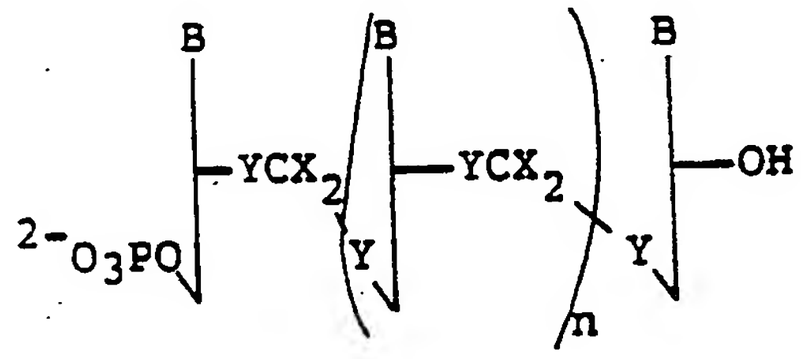
For ease in representation, the structure of the "wild type" oligonucleotides (and their derivatives as defined above) will sometimes be shown in the following shorthand form:



In this representation, B represents the usual purine or pyrimidine bases such as A, T, C, G as well as any modified forms of these bases that might be desirable in a particular therapeutic or compound. It is also understood that for some particular applications, one or more of these "B" groups may be replaced by any arbitrary substituent so long as that substituent does not interfere with the desired behavior of the oligomer. In

most cases, these may be replaced by analogous substituents as defined above.

The corresponding shorthand for the modified oligomers of the invention is, similarly,

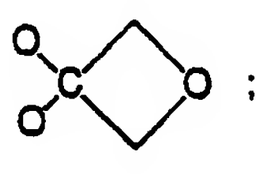


where similar remarks apply to the nature of B and to the possibility of derivatization. In addition, as the linkages of the invention are stable to nuclease attack, in addition to modified forms of DNA, similarly modified forms of RNA are included in the invention.

It should be clear that the invention compounds are not limited to oligomers of homogeneous linkage type, and that alternating or randomly distributed phospho-diester (or other substituted) and formacetal/ketal type linkages are contemplated. Since the oligomers of the invention can be synthesized one nucleotide residue at a time, each individual linkage, and the nature of each individual "B" substituent can be chosen at will. However, for most applications, it is contemplated that major portions of the oligomer designed for therapeutic use would contain the formacetal/ketal type replacement linkage so as to minimize those portions of the molecules subject to attack by nucleases and to enhance cell permeation.

X must be of such a nature so as to be stabilizing of the substitute invention link. For embodiments wherein both Y are O- i.e. $-OCX_2O-$; alkyl

substituents are unworkable unless one X attached to the ketal C is compensated by a high electron withdrawing ability of the other. A preferred embodiment for X in this case is simply H-; this has sufficient electron-withdrawing characteristics to result in adequate stability. Other preferred embodiments of X include carboxyl or the esters or amides thereof; halide, including F, Cl, Br and I; NO_2; phosphate or phosphorate at low pH; -SOR or $-SO_2R$ wherein R' is an organic residue, typically alkyl (1-6C); or is -CN or $-CF_3$. Other embodiments include electron withdrawing groups separated from the linking C of $-OCX_2O-$ by 1-3 methylene groups; i.e., X is $-(CH_2)_nW$ wherein n is 1-3 and W an electron withdrawing substituent. In general, the greater the value of n, the more electron withdrawing W must be. Typical embodiments of W include primary, secondary, or tertiary amines, or W further contains a heteroatom, such as hydroxymethyl, or substituted hydroxymethyl, such as OCH_2OCH_2OH, or e.g., OCH_2CH_2OH. In addition, both X together may constitute a cyclic substituent containing at least one heteroatom. In these embodiments, the ketal linkage may thus be, for example,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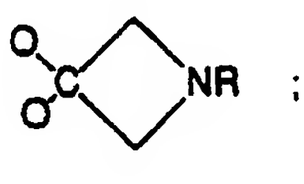
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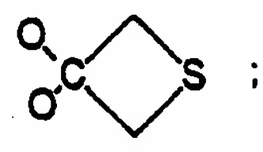
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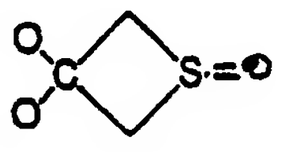

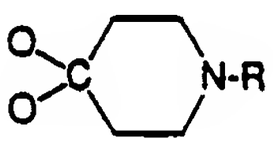
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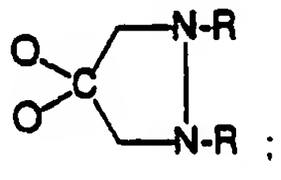
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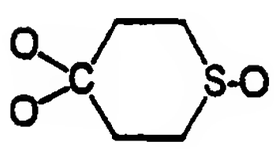
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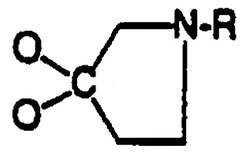
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Suitable X substituents include, therefore, -H, -F, -Cl, -Br, $-NO_2$, $-SCH_2CH_3$, -COOH, $-COOCH_3$, $-COOCH(CH_3)_2$, $-CONHCH_3$, $-CH_2F$, $-CF_3$, $-CH_2COOCH_3$, $-CH_2CONHCH_2CH_3$, $-CH_2CH_2COOH$, $-CH_2CH_2NH_2$, $-CH_2CH_2NHCH_2CH_3$, $-CH_2CH_2CF_3$, and so forth. If the Xs attached to the ketal linkage are different, both Xs together must confer sufficient electron withdrawing ability to stabilize the oligomer.

Formacetal/ketal type linkages wherein one or more of the linking oxygens is replaced by a sulfur are inherently more stable than their oxygen containing analogs. Thus, linkages of the formula $-OCX_2S-$, $-SCX_2O-$, and $-SCX_2S-$, are less demanding of electron withdrawal than the linkage $-OCX_2O-$. Accordingly, all of the embodiments set forth above for X in the context of the formacetal/ketal linkage are suitable, but in addition, X may also be alkyl, in general, lower alkyl (1-4C) or even phenyl, so long as the phenyl contains an electron withdrawing substituent in the appropriate position--e.g., 2-nitro, 4-nitro or 2,4-nitrophenyl. Of course, the linkage $-SCX_2S-$ is the most tolerant of electron donation. The extent to which the spectrum of substituents for X can be extended into those which are potentially electron donating can readily be determined by simple assays of stability of the resulting product, and this determination, and a good deal of predictability of the tolerance of these linkages, is within the ordinary skill of the art.

It should further be noted that if X, itself, contains a functional group, X can be used to tether desired moieties useful as adjuncts in therapy, for example, intercalators or minor groove reactive materials, such as netropsin and its derivatives, anthramycin, quinoxaline antibiotics, actinomycin, and pyrrolo (1-4) benzodiazepine derivatives.

The oligomers of the invention may contain an arbitrary member of the formacetal/ketal type linkages of the invention. These may be identical to each other or different by virtue of the embodiments chosen for X, and/or by virtue of the choice of $-OCX_2O-$, $-SCX_2O-$, $-OCX_2S-$ or $-SCX_2S-$. Since the oligomers are prepared sequentially, any pattern of linkage types, base substituents, and saccharide residues may be used.

In some preferred embodiments, the formacetal/ketal type linkages alternate in a regular pattern. Preferred embodiments include oligomers which contain, for example, a series of 3-8, formacetal type linkages alternating regularly with respect to regions of diester linkages. Particularly preferred are alternating structures wherein a formacetal type linkage is followed by a phosphodiester followed by a formacetal followed by a phosphodiester, etc., so that there is a one-by-one alternation of the two types of linkages. Additional alternatives might include, for example, a formacetal type linkage followed by two diesters followed by a single formacetal type linkage followed by two diesters, etc. A variety of regularly variant patterns is readily derived.

It is also clear that arbitrary modifications may be made to one or more of these saccharide residues; however, for the most part, the standard 3'-5' nucleotide linkage between ribosyl residues is most convenient. Where this is the case, further abbreviation of the

structures may be used. For example, in standard DNA (or RNA) the sequences are generally denoted by the sequence of bases alone, such as, for example, ATG CGC TGA. In general, it is simply stated in advance whether this represents an RNA or DNA sequence. In the compounds of the invention, similar notation will be used for modifications of otherwise physiological DNA or RNA molecules but the phosphodiester linkages replaced by the formacetal/ketal type will be noted in the structure. Thus, 5'-ATG (OCX_2O) GTCA (SCX_2O) AGG-3' indicates an oligonucleotide ATGGTCAAGG with two of the phosphodiester linkages modified in the noted positions.

The modified oligomers of the invention are, as stated above, useful in applications in antisense therapy. The specific targets of such therapies include: viral diseases, malignant cell growth, bacterial diseases, and, in fact, any condition associated with the presence of a characteristic DNA or RNA or products thereof. The compounds of the invention can be applied in the same manner as alternative modified oligonucleotide analogs, and the manner of such application is conventional in the art.

B. Utility and Administration

Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic and research contexts. In therapeutic applications, the oligomers are utilized in a manner appropriate for antisense therapy in general--as described above, antisense therapy as used herein includes targeting a specific DNA or RNA sequence through complementarity or through any other specific binding means, for example, sequence-specific orientation in the major groove of the DNA double-helix, or any other specific binding mode. Other biological entities, such as proteins, can also be

targeted. For such therapy, the oligomers of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Meade Publishing Co., Easton, PA, latest edition.

For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the oligomers of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays, for example, or using suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics.

For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams, as is generally known in the art.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA or RNA

sequences to which they specifically bind. Such diagnostic tests are conducted by hybridization through base complementarity or triple helix formation which is then detected by conventional means. For example, the oligomers may be labeled using radioactive, fluorescent, or chromogenic labels and the presence of label bound to solid support detected. Alternatively, the presence of a double or triple helix may be detected by antibodies which specifically recognize these forms. Means for conducting assays using such oligomers as probes are generally known.

In addition to the foregoing uses, the ability of the oligomers to inhibit gene expression can be verified in in vitro systems by measuring the levels of expression in recombinant systems.

It may be commented that the mechanism by which the specifically-binding oligomers of the invention interfere with or inhibit the activity of a target RNA or DNA is not always established, and is not a part of the invention. If the oligomer seeks, for example, a target mRNA, translation may be inhibited. In addition, by binding the target, the degradation of the mRNA message may be enhanced, or the further processing of the RNA may be inhibited. By formation of a triple helix, the transcription or replication of the subject DNA may be inhibited; furthermore, reverse transcription of infectious RNA or replication of infectious DNA is interfered with. It is also thought that the immune function may be modulated through physiological mechanisms similar to those induced by double-stranded RNA as exemplified by the "ampligen" system or similar to those used to suppress systemic lupus erythematosus. The oligomers of the invention are characterized by their ability to target specific oligonucleotide sequences regardless of the mechanisms of targeting or the

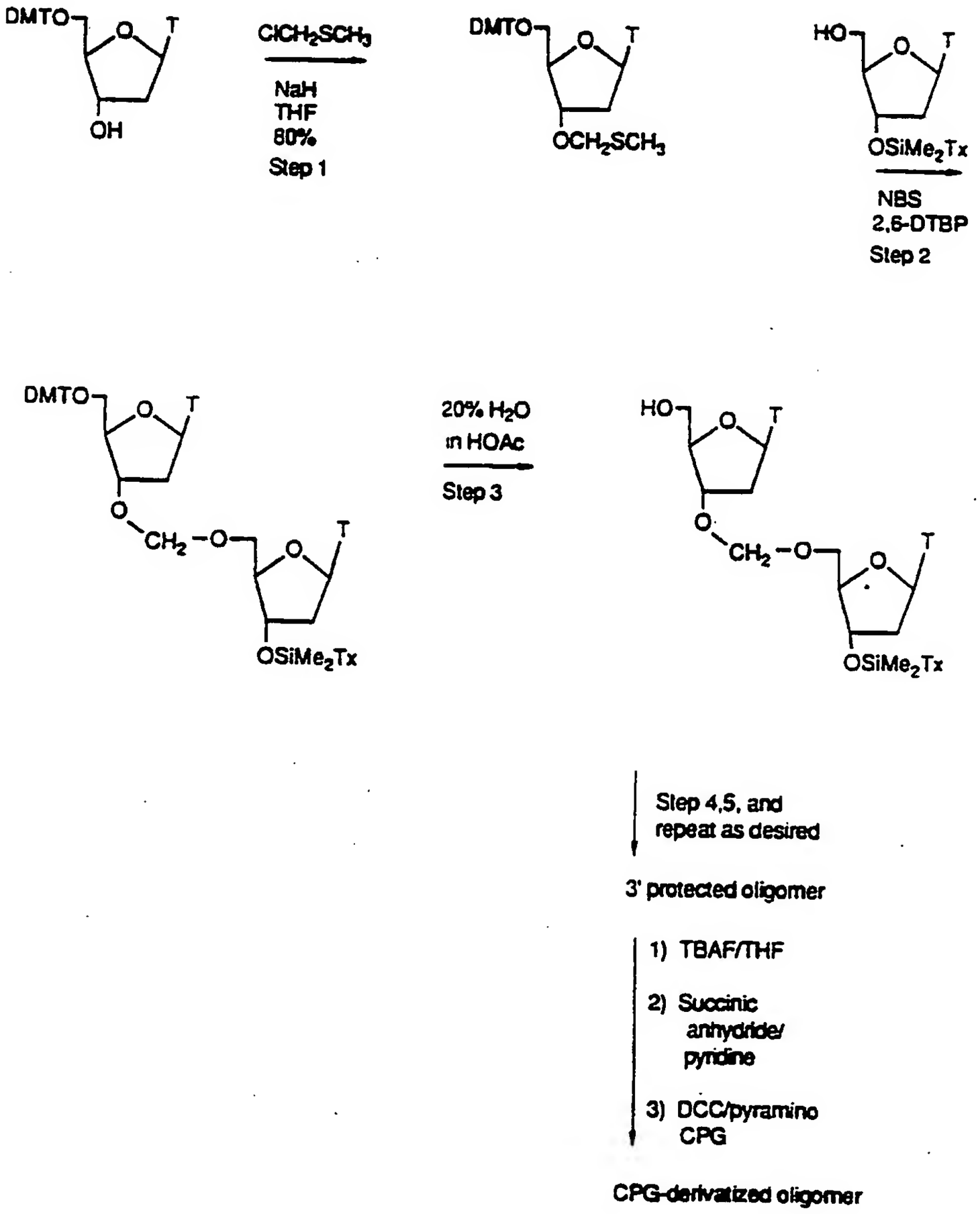
mechanism of the effect thereof. The foregoing is equally applicable to use of the oligomers to target other biological molecules.

Finally, it is understood that the DNA can be derivatized to a variety of moieties which include, intercalators, chelators, lipophilic groups, label, or any other substituent which modifies but does not materially destroy the oligomeric character of the backbone.

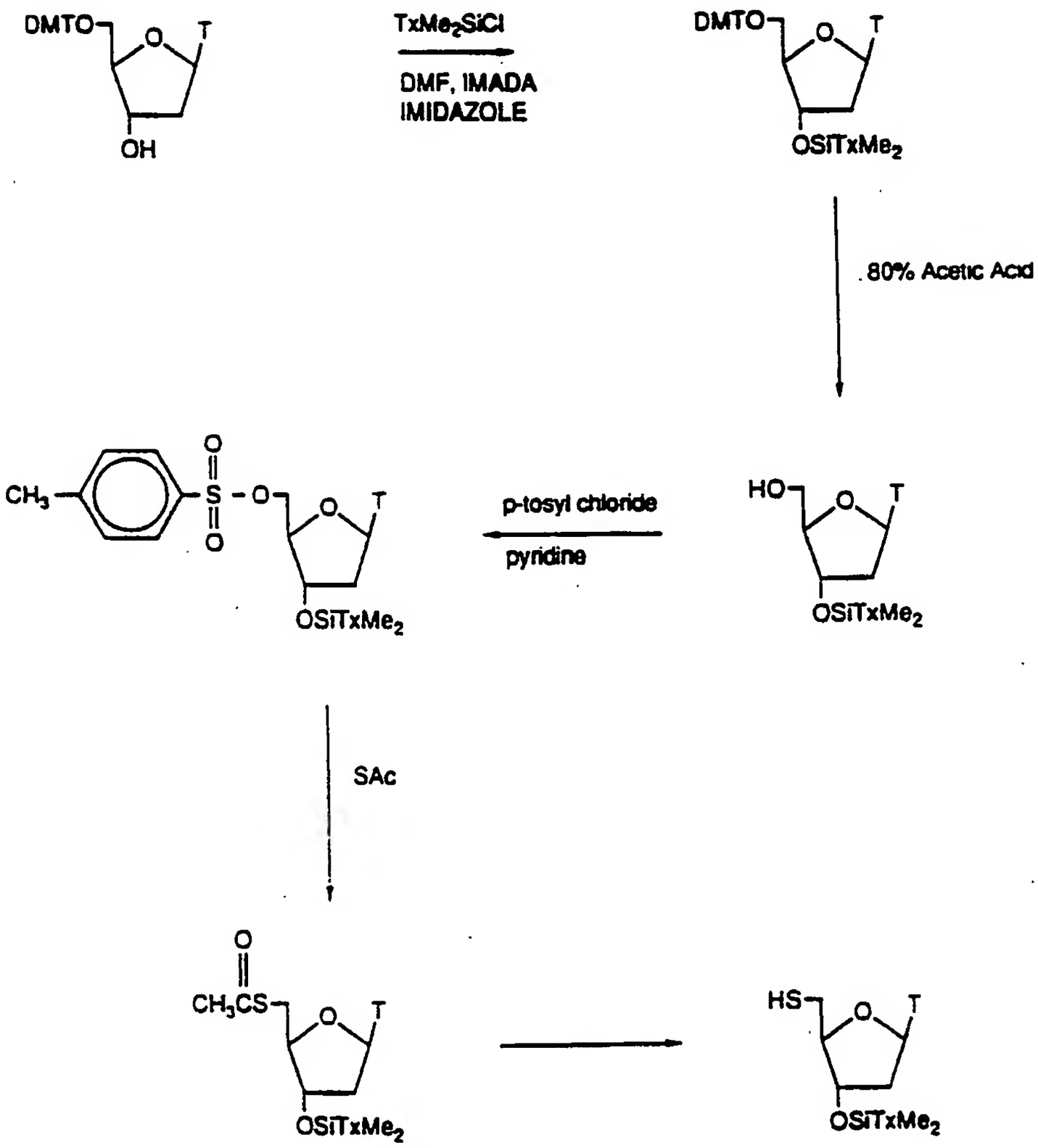
C. Synthesis of the Analogs

The oligomers (including dimers and trimers) of the invention which contain substituted formacetal/ketal linkages are coupled using a modification of the disaccharide synthesis method of Nicolaou, K.C., et al., J Am Chem Soc (1983) 105:2430. The sugar derivatized monosaccharides to be coupled are protected in the positions where participation in the linkage is not desired; one partner is treated with a reagent to form a thioacetal intermediate, such as Cl-CX_2SMe in the presence of a base and in an inert solvent to obtain the methyl thioacetal derivative at the free hydroxyl. This activated sugar is then reacted with the saccharide to which it is to be coupled, said saccharide protected at all positions other than the hydroxyl intended to form the linkage. This reaction is conducted in the presence of N-bromosuccinimide and a sterically hindered weak base to obtain the protected dimer. A suitably deprotected dimer can then be extended by repeating this conjugation. The use of this general approach to the formation of a formacetal linked trimer is shown in Reaction Scheme 1.

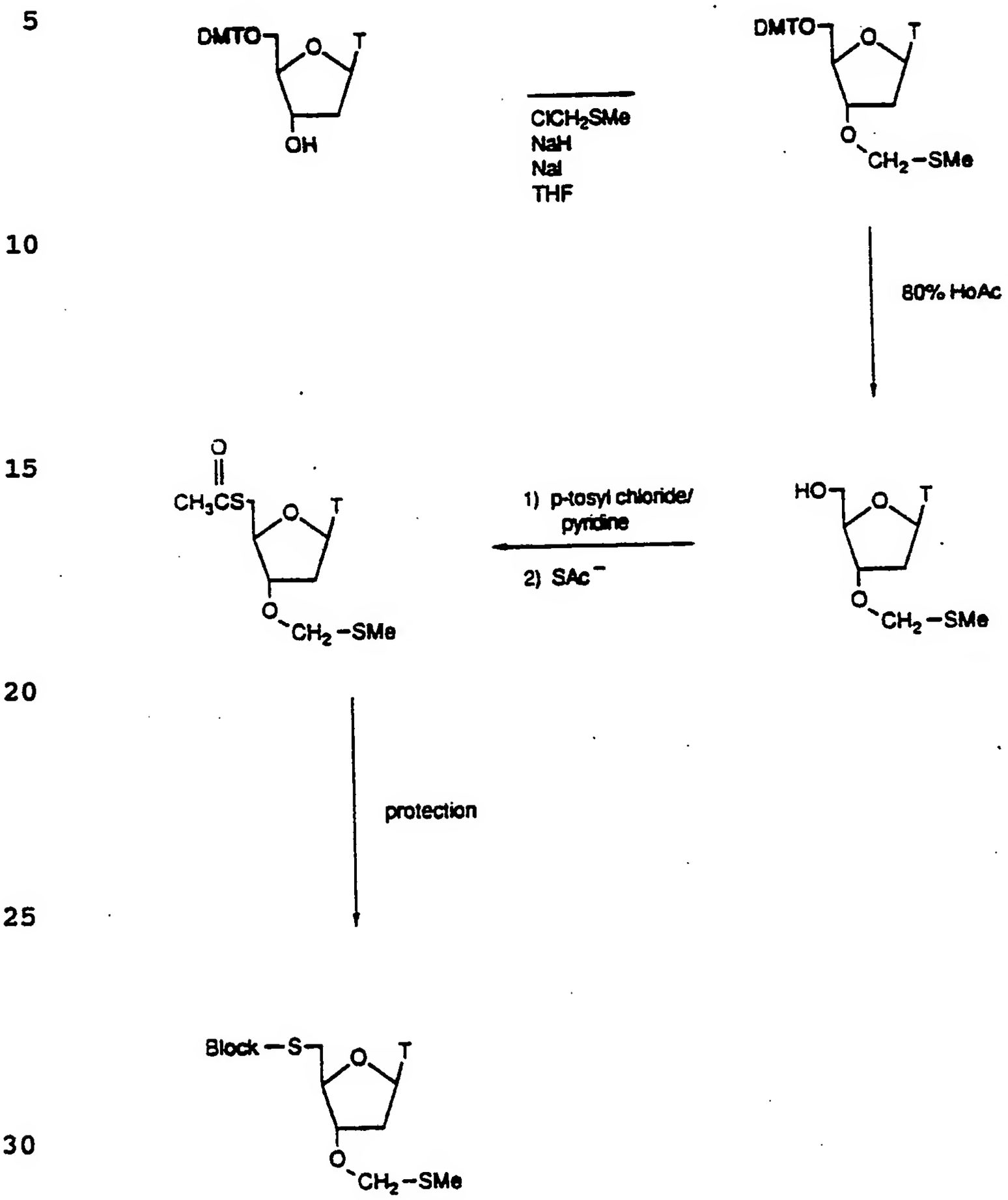
Reaction Scheme 1



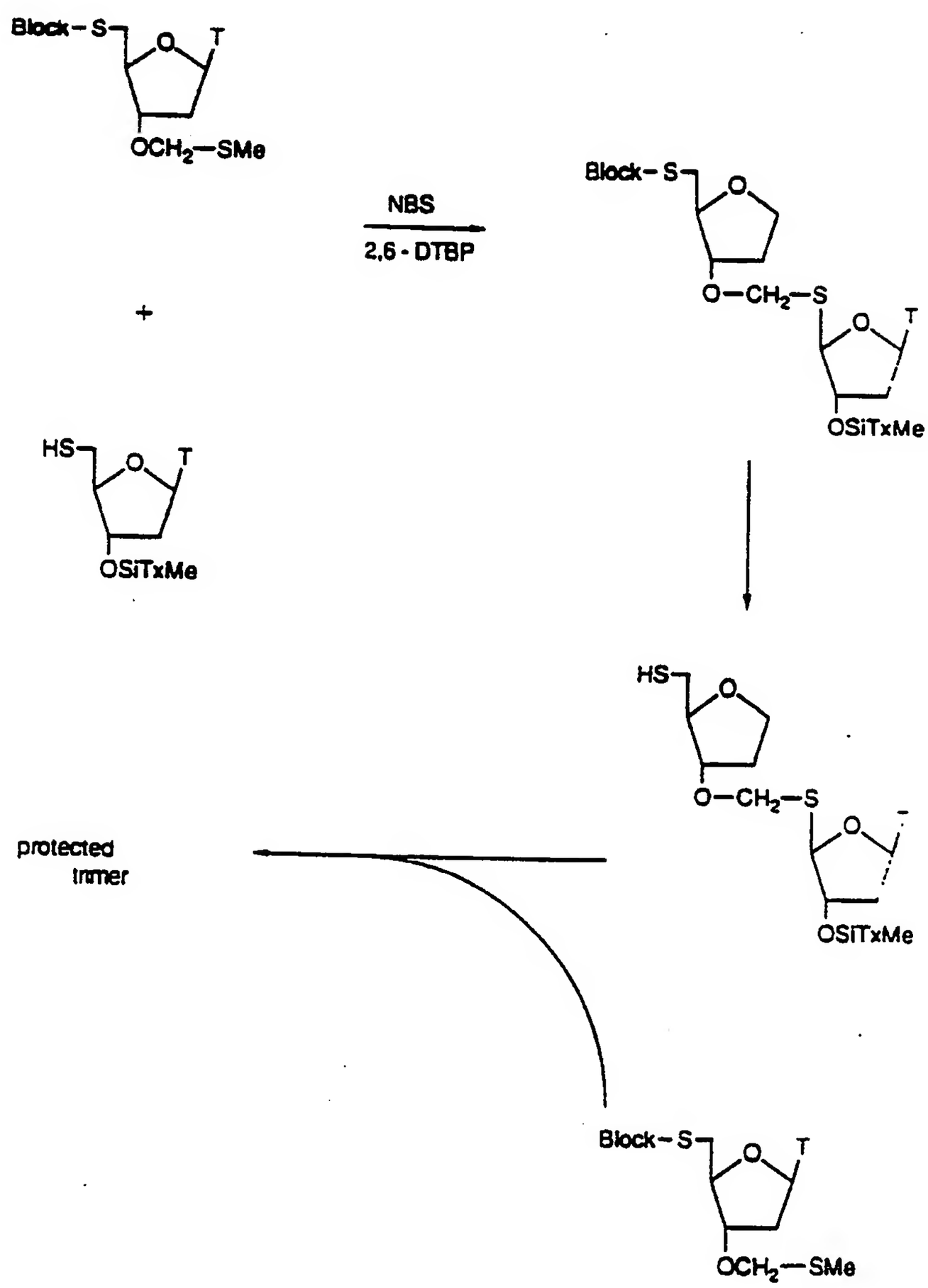
Reaction Scheme 2



Reaction Scheme 3



Reaction Scheme 4



The thymidyl derivatives of deoxyribose are used for illustration. In step 1, thymidine protected at the 5' position with dimethoxytrityl (DMT) is converted to the methyl thioacetal or other thioacetal. The $ClCH_2SCH_3$ reagent shown may be replaced by the corresponding bromide, or other thioethers of the general formula $ClCX_2SR$ or $BrCX_2SR$. The reaction in step 2 is conducted in the presence of N-bromosuccinimide (NBS) and 2,6-di-t-butylpyridine (2,6 DTBP) in methylene chloride for reaction of the thioacetal with a 5' hydroxyl of thymidine protected at the 3' position with thexyl dimethylsilyl. It should be noted that the hindered pyridine, 2,6-t-butylpyridine is required in step 2 since the less hindered dimethyl form is alkylated by the activated acetal. This results in the protected dimer containing the desired formacetal/ketal linkage. Deprotection in acid liberates the 5' position from the protecting trityl group, providing a dimer which can then be reacted with the 5'-DMT-3'- methylthioacetal as in step 2 in an additional step 4 to obtain the protected trimer, which can be deprotected as in step 3 in an additional step 5.

These steps can be repeated choosing any derivatized sugar for the 5'-protected 3'-derivatized reagent in a reaction analogous to step 2 to obtain oligomers of any desired length.

Reaction Schemes 2, 3 and 4 show the production of dimers and trimers which have linkages of the form $3'\text{-}OCX_2S\text{-}5'$. Reaction Schemes 2 and 3 show the preparation of the individual components of the dimer. Thymidine derivatives are used in this example. Reaction Scheme 2 describes the preparation of the 3'-protected thymidine having SH in the 5' position. As shown in the sequence of reactions, a 5' dimethoxytrityl thymidine is silylated using thexyl dimethylsilyl chloride. The

resulting 3'-silylated sugar is hydrolyzed in 80% acetic acid and the 5'-hydroxy is then activated using p-tolylsulfonyl chloride in pyridine; the p-tolylsulfonyl group is then replaced using a salt of thioacetic acid as described by Reist, E., et al., J Am Chem Soc (1964) 29:554. The acetyl thioester can be stored, and is hydrolyzed immediately prior to use to prevent disulfide formation, as described in the Reist paper.

Reaction Scheme 3 describes the activation of the 3'OH subsequent to conversion of the 5' position to a protected sulfhydryl, if desired. As shown, the 5'dimethoxytrityl protected thymidine is alkylated with chloromethylthiomethane in THF using sodium hydride as a base and sodium iodide as a catalyst. To obtain the 3'thioacetal other reagents besides chloromethylthio-methane may be used, such as chloromethylthiobenzene and chloromethyl-2-thiopyridine, or the corresponding bromides of any of the above. These may even be superior or lead to thioacetal products which have leaving groups superior to the methylthio shown. The protecting dimethoxytrityl group is then removed with 80% acetic acid, for example, and activated and converted to the thioester as was described in Reaction Scheme 2-- activation with tosyl chloride, followed by treatment with a salt of thioacetic acid.

The acetyl thioester may itself subsequently be used in the condensation reaction with the product of Reaction Scheme 2, but superior methods for protection of the SH are available. In one such scheme, the acetylthio group is hydrolyzed and the resulting sulfhydryl reacted with dimethoxytrityl chloride or trimethoxytrityl chloride generating a trityl sulfide, according to the procedure of Hiskey, R.G., et al., J Org Chem (1966) 31:1188. Subsequent deprotection is effected under mild acid conditions. In a second approach, the hydrolyzed

free SH group is reacted with dinitrophenol sulfonyl chloride, thus generating a disulfide group, according to the method of Fontana, A., J Chem Soc Chem Commun (1975) 976. The disulfide can be removed with reducing agents such as mercaptoethanol or sodium borohydride. Both of these alternative schemes of protection result in a visible color when deprotection is conducted.

The protected thioacetal monomer resulting from scheme 3 is then reacted with the 3'-protected 5' sulfhydryl product of Reaction Scheme 2 as shown in Reaction Scheme 4. The components are mixed in slightly less than an equivalent of n-bromosuccinimide in the presence of an excess of a hindered base, such as 2, 6-di-t-butyl pyridine as shown. The NBS and 2,6-DTBP are first added to the blocked thioacetal and the activated 3' thioacetal thymidine is then reacted with the 5' SH thymidine. This order of addition is required to prevent the side reaction of the reagents with the 5'SH group. The resulting dimer can be extended as shown in Reaction Scheme 4 by repeating the condensation reaction with another equivalent of the thioacetal.

The 3' thionucleosides are also available in the art, and can be synthesized as described by Zuckermann, R., et al., Nucl Acids Res (1985) 15:5305, and 3' thiothymidine has been used for the synthesis of oligonucleotides as described by Cosstick, R., et al., Tet Lett (1989) 30:4693-4696, cited above. The 3' thionucleosides can be converted to the dithio analogs of the acetals in a manner analogous to the first steps of Reaction Scheme 1 or Reaction Scheme 3 to provide a suitable leaving group for nucleophilic attack by either an SH or OH at the 5' position of the adjacent nucleoside, in a manner analogous to that shown in Reaction Scheme 1 or 4. Thus, in a manner analogous to the methods shown for the preparation of the

formacetal/ketal and the linkage of the formula $-OCX_2S-$, dimers, trimers, and oligomers with linkages of the formulas $-SCX_2O-$ and $-SCX_2S-$ can be obtained.

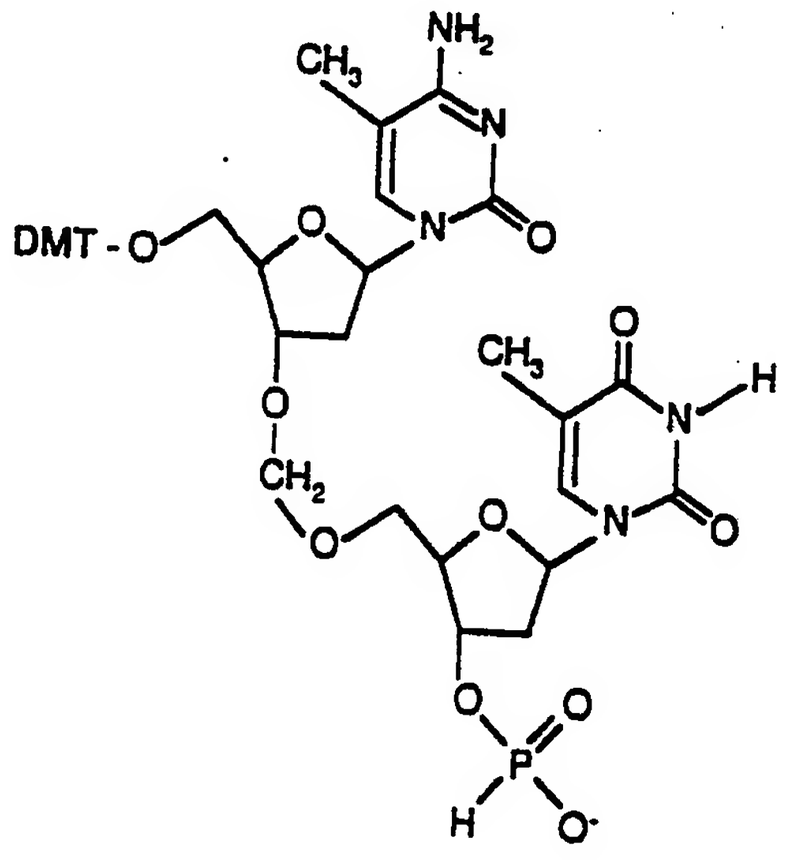
If desired, the resulting dimer, trimer or oligomer may be desilylated with tetrabutyl ammonium fluoride (TBAF) in an inert solvent such as THF and succinylated as a convenient linker for coupling to a solid support, such as controlled pore glass (CPG). The coupled modified oligomer can be used as a starting material for standard oligonucleotide synthesis, as, for example, using H-phosphonate chemistry as described by Froehler, B., et al., Nucleic Acids Res (1986) 14:5399. This synthesis involves deprotection of the 5' hydroxyl using dichloroacetic acid in methylene chloride and treatment with a 5' DMT-protected base 3' phosphonate in the presence of acetyl chloride/pyrimidine/acetonitrile, and repetition of this deprotection and linkage protocol for any desired number of times.

Alternatively, the liberated 3'OH can be linked via an ester linkage to a solid support analogous to standard oligonucleotide synthesis (Matteucci, M., et al., J Am Chem Soc (1981) 103:3185, for extension of oligonucleotide.

Additional formacetal/ketal type linkages can be inserted in the oligomer by phosphorylating the dimers, trimers, pentamers, etc., obtained as set forth in Reaction Schemes 1-4 and using these as the adducts in the oligomeric chain extensions. The final product is removed from the solid support by standard procedures, such as treatment with iodine in a basic aqueous medium containing THF or other inert solvent, followed by treatment with ammonium hydroxide. Deprotection of the nucleotide bases attached to the added nucleotides is also conducted by standard procedures.

The formacetal/ketal type linkage can be included at any arbitrary position in an oligonucleotide by substituting for a conventional monomer in the sequential synthesis, a protected dimer containing the formacetal/ketal type linkage which has been synthesized, for example, by steps 1-3 set forth in Reaction Scheme 1, or in Reaction Scheme 4, shown above, except that TBAF followed by phosphorylation (Froehler, B., et al., supra) is substituted for the hydrolysis/deprotection reagent in step 3 of Scheme 1 or the dimer of Reaction Scheme 4 is thus deprotected, so that the 3' position from which the protective group is removed is phosphorylated. The phosphate is conveniently present as the triethylamine salt. This dimer is then incorporated at any desired location in the synthetic process.

Thus, synthesis of the oligomer using standard techniques can be effected using the formacetal dimer synthons, for example, of the formula:



Formula A

Utilization of a series of these synthons results in the one-by-one alternation of the formacetal and phosphodiester linkages. Extended regions containing phosphodiester linkages are easily included by extending the chain with single nucleotides.

Any DNA synthesis chemistry such as phosphoramidate or phosphonate chemistry can be used to link monomers or dimers in a manner analogous to that set forth above.

The following examples are intended to illustrate but not to limit the invention:

Example 1

Preparation of 5' TCTCCCTCTCTTT(OCH$_2$O)T(OCH$_2$O)T-3'

See also: Matteucci, M.D., Tet Lett (1990) 31:2385.

Part A: Oligomer Preparation

Preparation of 5' Dimethoxytrityl 3'-Methylthiomethyl thymidine (Step 1):

Dimethoxytrityl thymidine (Peninsula Labs) (0.94g) was rotovaped from pyridine two times and then from methylene chloride/toluene one time. The resulting foam was dissolved in 75 ml dry CH_2Cl_2 and 10 ml of dry THF. 10 mg of NaI and .21g of NaH (60% dispersion in oil) was added under an argon atmosphere with stirring at 20°C. After 15 min the H_2 bubbling stopped and the reaction was stirred for another hour. Chloromethylthiomethane (158 μl) was added and stirring under argon continued for 16 hrs at 20°C. The reaction was quenched with 1M $NaHCO_3$, extracted and the organic layer dried with Na_2SO_4. The solution was then rotovaped to a foam and purified on a silica gel column using 5% isopropyl alcohol/CH_2Cl_2 as the eluent. Fractions were

concentrated to a pure foam (yield 0.56g) ^1H NMR spectra was consistent with assigned structure.

Preparation of 3' Thexyldimethylsilyl thymidine:

Dimethoxytrityl thymidine (Peninsula Labs) (1g), 20 ml of dry DMF, 193 mg of imidazole and 0.45 ml of thexyldimethylsilylchloride were combined under argon and stirred for 16 hr at 20°C. The reaction was then quenched with 2ml MeOH and rotovaped to a foam, dissolved in EtOH (20 ml) and treated with 0.6 ml of dichloroacetic acid for 16 hr at 20°C. The reaction was quenched with 1M $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was dried with Na_2SO_4. This solution was rotovaped to a foam, dissolved in 5 ml of diethylether and precipitated into 50 ml of hexane. The ether was rotovaped off as the white precipitate became heavy and upon filtration and drying, 0.5g of pure white foam was recovered. The ^1H NMR spectra was consistent with the assigned structure.

Preparation of 5' OH 3' Thexyldimethylsilyl thymidine formacetal thymidine (Steps 2 and 3):

5' dimethoxytrityl 3' methylthiomethyl thymidine (78 mg) and 3' thexyldimethylsilyl thymidine (60 mg) were rotovaped from toluene and dissolved in 2 ml of dry toluene. 50 mg of activated 4A molecular sieves and Di-t-butyl pyridine (0.144 ml) were added and the reaction was stirred for 16 hr under argon. N-Bromo-succinimide (13 mg) was then added and the reaction stirred for 1 hr at 20°C. The reaction was quenched with 1M $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was dried with Na_2SO_4. The solution was rotovaped to a foam and then treated with 4 ml of 20% H_2O/HOAc for 2hr at 20°C. The reaction was quenched in 1M $NaHCO_3$, extracted with CH_2Cl_2, dried with Na_2SO_4 and rotovaped to

a foam. The foam was purified by silica gel chromatography using 3% to 10% IsOH/CH_2Cl_2 as the eluent yielding 60 mgs of pure product after evaporation of solvent. The ^1H NMR spectra was consistent with the assigned structure.

Preparation of 5' Dimethoxytrityl 3' OH thymidine formacetal thymidine formacetal thymidine (Step 3):

5' OH 3' thexyldimethylsilyl thymidine formacetal thymidine (55 mg) was combined with dimethoxytrityl 3' methylthiomethyl thymidine (78 mg) and rotovaped from toluene. The resulting foam was dissolved in 2 ml of toluene/CH_2Cl_2 (1/1), 50 mg of activated 4A molecular sieves and di-t-butylpyridine (0.144 ml) were added and the reaction was stirred for 16 hr under argon. N-Bromosuccinimide (21 mg) was then added and the reaction stirred for 1 hr at 20°C. The reaction was quenched with 1M $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was dried from Na_2SO_4. The solution was rotovaped to a foam and treated with 1 ml of tetrabutylammonium fluoride in THF for 3 hr, extracted, dried and evaporated in normal fashion. The resulting foam was purified by preparative silica gel thin layer chromatography using a 10% IsOH/CH_2Cl_2 as the eluent. The ^1H NMR was consistent with the desired structure (yield: 25 mg). A small aliquot (5 mg) was deprotected at the 5' end in normal fashion.

The resulting 5' OH 3' OH thymidine formacetal thymidine formacetal thymidine was used in further characterization. ^1H NMR of this material in d_6 DMSO was consistent with the assigned structure. An aliquot of this was persilylated and a Fast Atom Bombardment Mass Spectrum was recorded. This confirmed the desired molecular weight. Additionally the di OH material was

used in the chemical and enzymatic stability studies previously mentioned.

Preparation of 5' Dimethoxytrityl 3' succinylate thymidine formacetal thymidine formacetal thymidine:

5' Dimethoxytrityl 3' OH thymidine formacetal thymidine formacetal thymidine (16 mg) was treated with 9 mg of succinic anhydride and a trace of dimethylamino pyridine in pyridine for 16 hr at 20°C. The reaction was then rotovaped and extracted with 30% BuOH/CH_2Cl_2 against H_2O. The organic layer was dried, rotovaped and purified on a silica gel column using 10% H_2O/CH_3CN as the eluent. Rotovaping yielded 15 mg of product.

Preparation of 5'-TCTCCCTCTCTTT(OCH$_2$O)T(OCH$_2$O)T-3':

The 3' succinylated 5'-DMT thymidine formacetal thymidine formacetal thymidine from the previous paragraph was then bound to solid support and deprotected. Standard procedures were used to extend the oligomer to obtain the title compound.

Part B: Stability to Acid

The purified product of paragraph (A) above was treated with 20% water/formic acid at 80°C for 1 hr, resulting in complete hydrolysis. However, when subjected to moderate acid treatment such as 1 M HCl at 20°C for 3 hrs or 20% water/acetic acid at 45°C for 3 hrs, no hydrolysis was observed.

The title compound was also stable to snake venom phosphodiesterase under standard conditions for hydrolysis of phosphodiester bonds.

Part C: Hybridization to Complementary RNA

An RNA sequence complementary to the title compound was generated using T7 transcription Milligan,

T.F., et al., Nucleic Acids Res (1987) 15:8783). This RNA was used to test the ability of the title compound to hybridize to its complement as compared to analogous sequences wherein the acetal linkages are replaced by phosphodiesters, phosphoramidates, or wherein the 3' terminal thymidyl nucleotides were deleted. The melting temperatures of complexes formed with the title compound and these controls were measured using 100 mM NaCl, 50 mM Tris, pH 7.5 under standard conditions as described by Summers, M.F., et al., Nucleic Acids Res (1986) 14:7421. The results are shown in Table 1, where "12mer" stands for the TCTCCCTCTCTT nucleotide sequence common to all of the samples. In the third oligomer, "W" represents the substituent $-CH_2CH_2OMe$ in the amidate.

Table 1

	Tm
12mer-T(OCH_2O)T(OCH_2O)T	59.0
12mer-TTT	59.5
12mer-T(PONHW)T(PONHW)T	58.5
12mer	56.5

As shown in Table 1, the three thymidines linked through formacetal linkages confer additional hybridization capability on the 12mer as compared to the 12mer alone. The additional hybridization is comparable to that conferred by extension by conventional phosphodiester linkages.

Part D: Binding to Duplex DNA

Using the footprinting method described in detail in Part C of Example 2 below, the formacetal-containing oligonucleotides of this example show ability to bind duplex DNA.

Example 2

Preparation of

5'TCTC*(OCH_2O)TC*(OCH_2O)TC*(OCH_2O)TC*(OCH_2OTTTT-3'

or 5'TCTC*·TC*·TC*·TC*·TTTT-3'

Part A: Preparation of Oligomers

A series of four oligomers as shown, wherein C* represents 5-methylcytosine and the dot between C* and T represents a linkage varied among the series of four oligomers was synthesized. In the "phosphodiester" control oligomer, the dot represents a conventional phosphodiester linkage. In the "methylphosphonate" oligomer, the dot represents a methylphosphonate linkage. In the "methoxyethyl amidate" oligomer, the dot represents a methoxyethyl amidate linkage. In the "formacetal" oligomers of the invention, the dot represents a formacetal linkage as described herein and as shown above.

The phosphodiester oligomer was synthesized using the conventional techniques described by Froehler, B.C., et al., Nucleic Acids Res (1986) 14:5399. The remaining three oligomers were synthesized using this method, but with the incorporation of the appropriate dimer synthon. The methylphosphonate and the methoxyethyl amidate dimer synthons were constructed using 5'-DMT-N-benzoyl-5-methyldeoxycytidine and 3-t-butyldimethylsilyl thymidine as described, respectively, by Miller, P.S., et al., Biochemistry (1986) 25:5092, and by Froehler, B.C., Tet Lett (1986) 27:5575. The formacetal dimer synthon was prepared as described hereinabove and as reported by Mateucci, M.C., et al., Tet Lett (1990) 31:2385, cited above. The oligos resulting from the synthesis were deblocked with concentrated ammonia for 16 hours at 20°C and gel purified using conventional techniques.

Part B: Hybridization to RNA Complement

The complementary RNA for use as a test compound was synthesized by T7 polymerase reaction on the appropriate primer template as described by Milligan, J.F., et al., Nucleic Acids Res (1987) 15:8783. Appropriate complementary DNA oligomers were also constructed.

The hybridization was tested using thermal denaturation comparisons in buffer containing salts designed to mimic intracellular salt composition and concentration--i.e., 140 mM KCl, 10 mM NaCl, 5 mM NaH_2PO_4, 5 mM $MgCl_2$, pH 7. UV absorption was measured in the temperature range 20°C-80°C, and all oligomers gave monophasic transitions. T_m values were assigned by finding the temperature at which the slope of the curve was maximum.

These results are shown in Table 2.

Table 2

Oligomer	T_m ssRNA	T_m ssDNA
Phosphodiester	60.0°C	49.5°C
Methyl Phosphonate	50.5	
Methoxyethyl Amidate	47.5	38.5
Formacetal	59.0	39.0

As shown in the table, the T_m of the formacetal oligomer in comparison to the phosphodiester is roughly equal for single-stranded RNA. On the other hand, the T_m values for the alternate analogs methylphosphonate and methoxyethyl amidate oligomers was appreciably lower. Thus, despite the advantages conferred by the formacetal linkage--for example, enhanced ability to cross cell membranes and enhanced nuclease resistance, no decrease

in the hybridization power to single-stranded RNA was detected.

Part C: Binding to Duplex DNA

The "footprint" assay described by Cooney, M., et al., Science (1988) 241:456, was used. The assay is based on the ability of the oligomer bound to duplex to protect the duplex from digestion with DNAse I. Various concentrations of the test oligomer ranging from 0.1-10 μM were incubated with a P^{32} radiolabeled restriction fragment bearing the target sequence at 1 nM concentration in 10 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 1 mM spermine and 20 mM MOPS buffer at pH 7 for 2 hours. The target sequence for the set of oligomers prepared in Part A of this example was

——————AGAGAGAGAGAAAA——————

——————TCTCTCTCTCTTTT—————— .

DNAse I was added to permit limited digestion, the samples were then denatured and subjected to polyacrylamide gel electrophoresis which separates the DNA fragments based on size.

An outline of the principle of the footprint assay and idealized results are shown in Figure 1. As shown in Figure 1, the labeled duplex, when treated with DNAse, should yield lengths of oligomer corresponding to cleavage at each diester linkage, thus obtaining the series of bands shown on the left in the idealized gel. On the other hand, when the duplex is protected by binding to the oligomer, the series of lengths represented by cleavage at the diester linkages in the region protected by binding to the oligomer is missing from the gel. This "footprint" indicates the region of protection. The results are semiquantitatively estimated

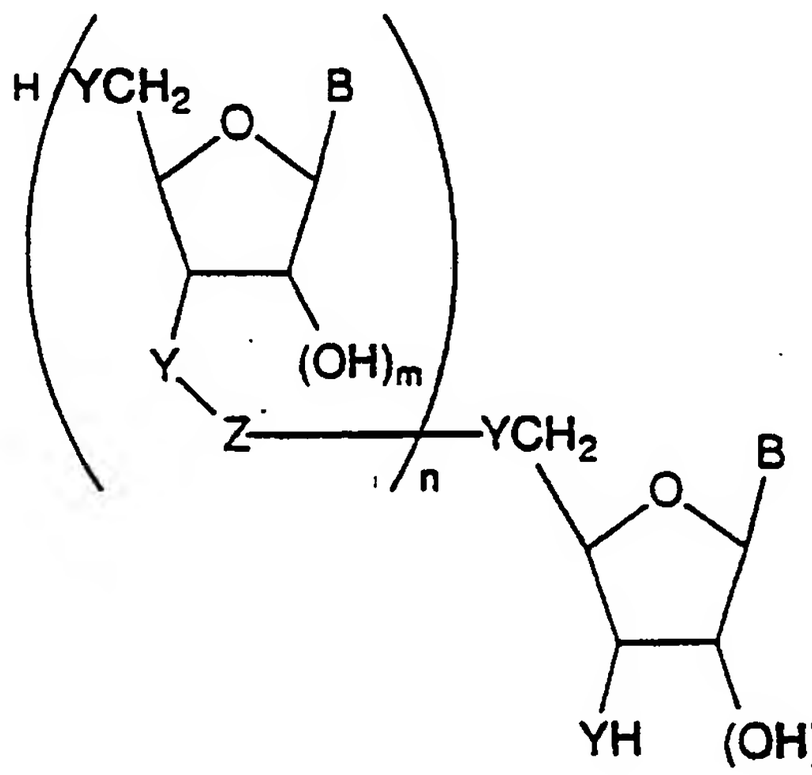
by observing either the complete absence of, or only weak appearance of, bands in the region of the footprint.

The formacetal oligomer and the phosphodiester oligomer showed partial protection of the target sequence at 0.1 μM and more than 90% protection at 1 μM concentration of the oligomer. On the other hand, the methoxyethyl phosphoramidate and the methylphosphonate showed only partial protection at 1 μM and >90% protection at 10 μM. Thus, these oligomers appeared to have markedly less affinity for the duplex than the formacetal oligomer.

Claims

1. A modified oligomer or derivative thereof, as herein defined, wherein said oligomer or derivative contains a nucleotide sequence useful in binding a biological moiety wherein the modification comprises substitution, for one or more linkages between individual nucleotide residues, of a linkage of the formula $-YCX_2Y-$ wherein each Y is independently O or S and wherein each X is independently chosen and is a stabilizing substituent, and the remaining linkages are phosphodiester linkages or conventional substitutes therefor.

2. An oligomer of the formula



(1)

or a derivative thereof as herein defined, wherein said oligomer or derivative contains a nucleotide sequence useful in binding a biological moiety,

wherein each B is independently a purine or pyrimidine residue or an analogous residue as herein defined, and

wherein each Z is independently

$-P(O)O^-$- of a phosphodiester conventional substitute therefor, as herein defined, or is

$-CX_2$- ; and

wherein each X is independently a stabilizing group, n is 1-200, each m is 0 or 1, and

wherein at least one Z is CX_2, and

wherein each Y is independently O or S.

3. The modified oligomer of claim 1 or 2 wherein both X included in a single $-YCX_2Y$- linkage are identical and/or wherein at least two linkages are said $-YCX_2Y$- linkages.

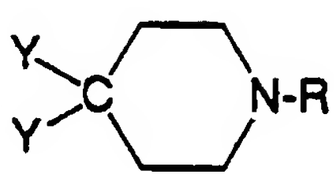
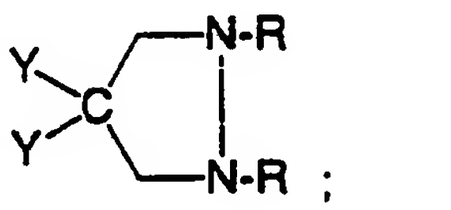
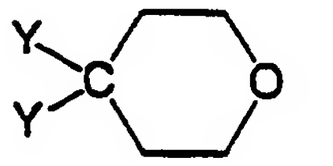
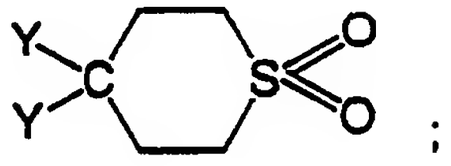
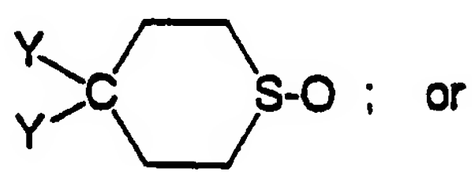
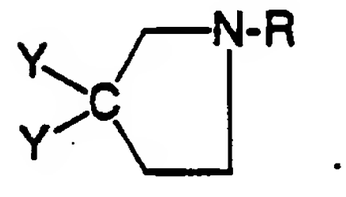
4. The modified oligomer of claim 3 which contains at least one region of at least four nucleotides wherein the $-YCX_2Y$- linkages and phosphodiester type linkages are alternating.

5. The modified oligomer of claim 1-4 wherein each X is selected from the group consisting of H; carboxyl or an ester or amide thereof; -SOR or $-SO_2R$, wherein R is alkyl(1-6C); -CN; and $-CF_3$; or X is $-(CH_2)_nW$ wherein n is an integer of 1-3 and W is selected from the group consisting of halo; carboxyl or an ester or amide thereof; phosphate or phosphonate or an ester thereof; -SOR or $-SO_2R$, wherein R is alkyl(1-6C); -CN; $-NO_2$; $-CF_3$; -OH, -OR, and a primary, secondary, or tertiary amine;

or both X taken together from a heterocyclic ring.

6. The modified oligomer of claim 1-4 wherein each X is H.

7. The oligomer of claim 5 wherein at least one $-YCX_2Y-$ is

8. The oligomer of claim 1-7 which is a dimer or trimer.

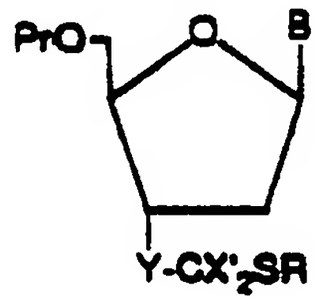
9. The oligomer of claim 1-8 wherein the conventional substitute for $-P(O)O-$ is selected from the group consisting of $P(O)S$, $P(O)NR_2$, $P(O)R$, $P(O)OR'$, CO, or CNR_2, wherein R is H or alkyl (1-6C) and R' is alkyl (1-6C).

10. The oligomer of claim 1-9 wherein the derivative comprises a conjugate with label, an intercalator, or a drug.

11. A method to synthesize a modified oligonucleotide containing at least one linkage of the formula $-YCX_2Y-$, which method comprises treating a compound of Formula 1 wherein n=1-3 and wherein all Z are CX_2 attached to a solid support with standard reagents for forming phosphodiester bonds to additional nucleotides.

12. A method to treat diseases mediated by the presence of a nucleotide or other biological DNA-binding moiety which comprises administering to a subject in need of such treatment an amount of the modified oligomer of claim 1-10 capable of specifically binding said moiety effective to inactivate said moiety.

13. A compound of the formula



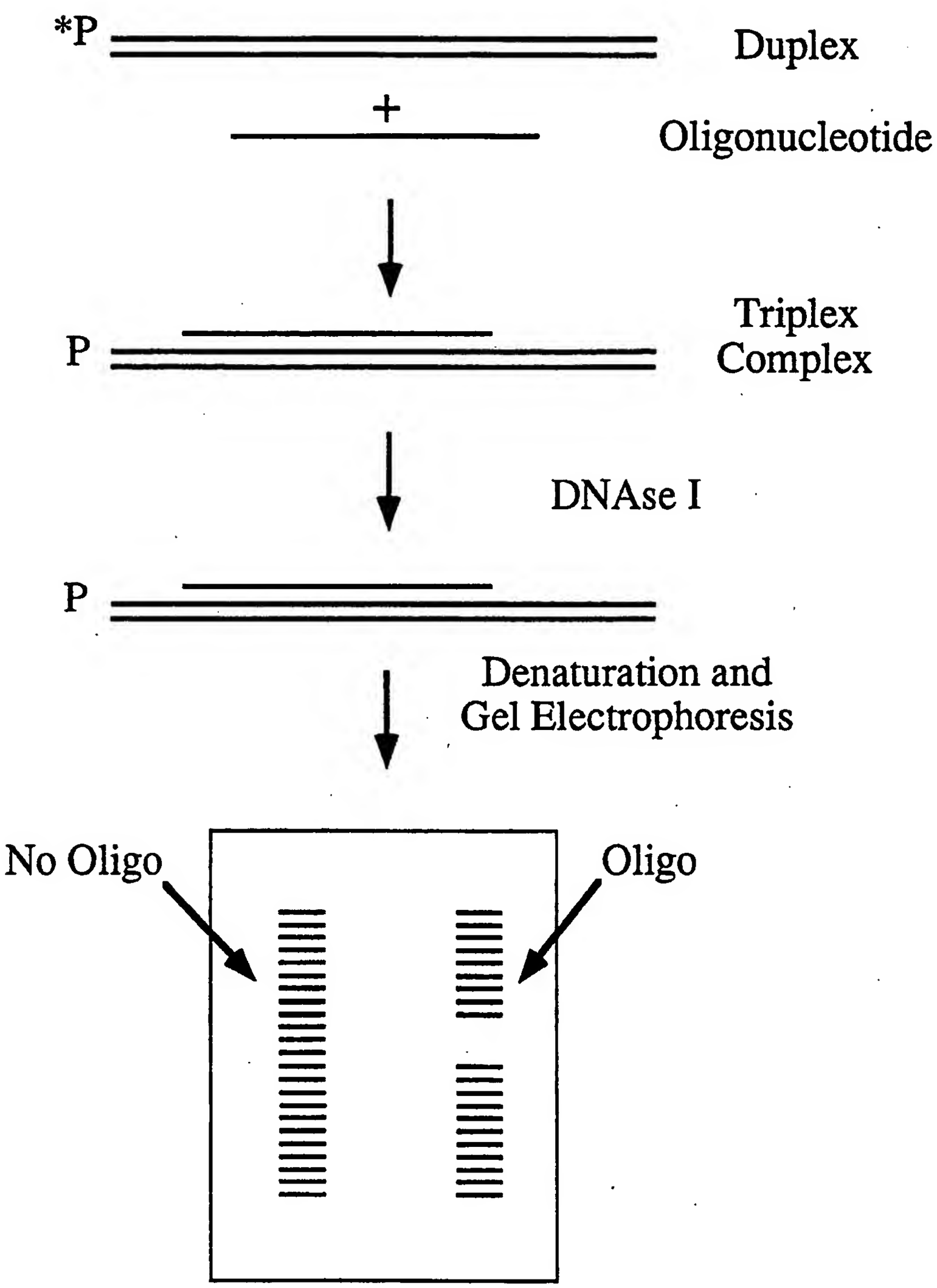
wherein Y is O or S;

B is a purine or pyrimidine residue or an analogous residue as herein defined;

each X' is independently a stabilizing group with the proviso that both X' cannot be H;

R is an aliphatic or aromatic hydrocarbyl radical which may further contain a heterocycle; and

Pr is H or a protecting group.


*P
Duplex
+
Oligonucleotide
Triplex
P
Complex
DNAse I
P
Denaturation and
Gel Electrophoresis
No Oligo
Oligo

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/06110

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 5/00.A61K 31/00

US. Cl.: 514/44; 536/27, 28, 29

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S. Cl.	514/44; 536/27, 28, 29

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,458,066 (CARUTHERS et al.) 03 July 1984, see entire document.	1-13
Y	WO, A, 89/05853 (TULLIS) 29 June 1989, see entire document.	1-13

* Special categories of cited documents: [15]

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
13 December 1990	20FEB 1991
International Searching Authority [1]	**Signature of Authorized Officer** [10]
ISA/US	James O. Wilson

Form PCT/ISA/210 (second sheet) (May 1986)

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